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Use of cell culture media for cultivation of the mite pathogenic fungi *Neozygites tanajoae* and *Neozygites floridana*

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Abstract

The pathogenic fungus *Neozygites tanajoae*, one of the most efficient natural enemies of the cassava green mite (CGM) *Mononychellus tanajoa* in Brazil, was introduced experimentally in Benin in 1998/1999 for the control of CGM. Isolation methods and culture media for in vitro production of *N. tanajoae* are reported for the first time in this study. Continuous growth of *N. tanajoae* was achieved using medium NT-1 (IPL-41 + 5–10% fetal bovine serum + 0.3% lactalbumin hydrolysate + 0.3% yeastolate). This medium supported production of *N. tanajoae* up to 1.53 (± 0.08) $\times 10^7$ hyphal bodies/mL after 8 days. The growth of *N. tanajoae* from Cruz das Almas, Brazil, was compared to the growth of two *Neozygites floridana* isolates with wider host ranges from North Carolina, US, and Palmira, Colombia, in 11 cell culture media. We demonstrated that differences in nutritional requirements exist between *N. tanajoae* and the similar species, *N. floridana*. *N. tanajoae* is a particularly fastidious species highly specific to CGM and grows well in few media while *N. floridana* which is less host specific, grows in a broader range of media, including serum free media. *N. floridana* isolates produced more than 2×10^6 hyphal bodies/mL in $\geqslant 7$ of the 11 media tested. However, the *N. tanajoae* isolate reached the same final concentration in only 3 media. Cell densities of *N. tanajoae* also increased slower than in *N. floridana* isolates in most media. *N. tanajoae* differed morphologically from the two *N. floridana* isolates in vitro. Hyphal bodies of eight *N. tanajoae* isolates are shorter than hyphal bodies of the two *N. floridana* isolates. The distinction of these two species was initially proposed based on host specificity, genetic and physiological patterns and is supported by the results presented in this study.

Keywords: Entomophthorales; *Mononychellus tanajoa*; *Tetranychus urticae*; Cell culture media; Isolation; Biological control

1. Introduction

Neozygites species (Zygomycetes: Entomophthorales) are among the most specialized of the Entomophthorales in terms of host specificity and growth requirements (Steinkraus, 1998). At present, few studies have been conducted on in vitro production of species in this genus, and only three species have been grown in vitro. Repeated attempts have been made to culture *Neozygites fresenii* (Gustafsson, 1965) and *Neozygites floridana* (Mietkiewski et al., 1993) in vitro without success. Over 400 attempts to culture *N. fresenii*, an important natural control agent of the cotton aphid, *Aphis gossypii*, in the USA, have been made by Steinkraus (1998). Delalibera Jr. (1996) and Leite et al. (2000) tried to isolate *Neozygites tanajoae* from Brazil (previously referred to as *Neozygites* sp. and *N. floridana*, respectively) but no continuous growth was achieved. Isolation and growth of protoplast-like cells of *N. tanajoae* was described by Delalibera Jr. (1996). However, those cells were probably abnormal vacuolated hyphal bodies generated by growth in an incomplete medium (I. Delalibera Jr., unpubl. data). Cultures were not established from that study, and no further investigations were undertaken to determine the nature of those cells.

Cultures of *Neozygites* have only been established using liquid cell culture medium and hyphal bodies from infected living hosts as the inoculum. Butt and Humber (1989) were the first to isolate *N. floridana* from

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twospotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae), using Grace's insect cell culture medium + 5% fetal bovine serum. Leite et al. (2000), using a modified version of Grace's medium supplemented with 0.33% lactalbumin hydrolysate + 0.33% yeastolate + 5% fetal bovine serum (TNMFH), were able to get higher yields of hyphal bodies from another isolate of *Neozygites* pathogenic to twospotted spider mite and cassava green mite, *Mononychellus tanajoa*, (CGM) (Acari: Tetranychidae) from Colombia. *Neozygites parvispora* isolated from *Thrips tabaci* is the only species of *Neozygites* pathogenic for hosts other than mites produced in vitro so far. Sustained growth of hyphal bodies of *N. parvispora* was achieved using Grace's medium supplemented with 10% pre-treated lepidopteran hemolymph and 20% fetal bovine serum (Grundschober et al., 1998).

Neozygites tanajoae is an important natural regulator of populations of the CGM in northeastern Brazil (Delalibera Jr. et al., 1992, 2000). This fungus was experimentally released in Africa in 1998/1999 for the control of the introduced CGM (Hountondji et al., 2002). One major problem in releasing exotic isolates of *N. tanajoae* in Africa is that a technique to distinguish strains of this fungus was not available. The absence of protocols to detect *N. tanajoae* and differentiate isolates released made it difficult to monitor the introduction of selected isolates to regions where the pathogen already exists. The ability to culture *N. tanajoae* in vitro would greatly facilitate genetic studies for development of molecular probes for strain detection. However, *N. tanajoae* did not grow in the media used for *N. floridana* isolated from *T. urticae* (Butt and Humber, 1989; Leite et al., 2000).

The objective of this study was to identify culture media to grow *N. tanajoae* in vitro and to compare the growth of *N. tanajoae* and *N. floridana* isolates in different media. We evaluated the in vitro growth of *N. tanajoae* in different liquid media used for production of obligatory entomopathogens and a variety of cell culture media and supplements. Initially, we supplemented different culture media with fetal bovine serum. Fetal bovine serum is very expensive and is unavailable or the supply is inconsistent in many developing countries where investigations of *Neozygites* are needed. Based on initial results, we evaluated alternative ingredients to replace fetal bovine serum, as well as testing mixtures of commercially available tissue culture media.

2. Materials and methods

2.1. Isolates

Isolates of *N. tanajoae* used for isolation were obtained by collecting infected cassava green mite in Brazil and Africa and were maintained in an in vivo collection at the Brazilian Organization for Agricultural Research (EMBRAPA)—Cassava and Tropical Fruits, Cruz das Almas, Bahia, Brazil. In vitro cultures of the two isolates pathogenic to *T. urticae* were from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF; Ithaca, NY, USA). Species included in this study, collection number and origin are listed in Table 1. Using the protocol described by Delalibera Jr. (2002) for in vivo production of *N. tanajoae* it was possible to generate a large number of infected mites for isolation and also to use isolates with very low viability due to long-term storage. Cultures of new isolates established were submitted to EMBRAPA—National Center for Genetic Resources, and Biotechnology Research, Brasilia, Brazil under Accession numbers CG866–CG873.

Table 1
Origin and host range of isolates of *N. tanajoae* and *N. floridana*

Location	Host range	Isolation	In vivo collection	In vitro collection
Garanhuns, PE, Brazil	CGM	30/03/99	BIN 30	
Cruz das Almas, BA, Brazil	CGM	31/03/99	BIN 8l13	CG 872
Cruz das Almas, BA, Brazil	CGM	30/06/00	BIN 8ddc	CG 866
Tianguá, CE, Brazil	CGM	16/07/00	BIN 10	CG 867
Cristinápolis, SE, Brazil	CGM	23/07/00	BIN 12	CG 868
Teotônio Vilela, AL, Brazil	CGM	23/06/00	BIN 14	CG 869
São M. dos Campos, AL, Brazil	CGM	23/06/00	BIN 15	CG 870
Gravatá, PE, Brazil	CGM	16/07/00	BIN 16	CG 871
Cotonou, Benin	CGM	20/07/00	BIN 35	CG 873
North Carolina, USA[a]	*T. urticae* and other tetranychid mites	18/10/81		ARSEF 662
Palmira, VA, Colombia[a]	*T. urticae* and CGM	12/96[b]		ARSEF 5376

ARSEF, Agricultural Research Service Collection of Entomopathogenic Fungal Cultures; BIN, Collection of *Neozygites* isolates—Embrapa Cassava and Tropical Fruits, Cruz das Almas, Brazil; CG, EMBRAPA—National Center for Genetic Resources, and Biotechnology Research, Brasilia, Brazil.

[a] *N. floridana*

[b] Date deposited in ARSEF collection.

2.2. Isolation and establishment of *N. tanajoae* in vitro

Isolations were carried out using mites that had been infected for 2 or 3 days. Infected CGM were placed in a 10 mL capped vial containing 1 mL of 0.5% sodium hypochlorite and mixed gently by inverting the tube for 3 min. The liquid was loaded into a sterile cell strainer (70 μm) inside a sterile hood. The strainer with the mites was then washed three times by dipping in autoclaved water. Each mite was individually transferred, using sterile toothpicks, to a 24-well tissue culture plate into 100–150 μl of the culture media being tested. Autoclaved toothpicks were used to open the mite body to release the hyphal bodies, and the mite removed. The culture plates were incubated at 23 ± 1 °C, in the dark. Fresh medium was added to the growing cultures as necessary. When the culture reached 500–1000 μl they were inoculated into 25 cm^2 culture flasks containing 3 mL of medium and incubated on an orbital shaker at 75 rpm at 20 °C.

In the first isolation attempts, Grace's insect cell culture medium (Gibco-BRL) was tested with the following supplements, individually or in different combinations: 5% fetal bovine serum + 0.3% lactalbumin hydrolysate + 0.3% yeastolate (all manufactured by Gibco-BRL), sucrose (10 g/L; Fisher Scientific), heart infusion (0.5–2%; Becton–Dickinson Microbiological Systems), tryptic soy broth (0.25–1%; Sigma Chemical), gypsy moth (*Lymantria dispar*) hemolymph (5–10%), and cassava leaf extract (0.5–5% of approximately 10 g of fresh leaves/100 mL H_2O). Late instars of gypsy moth larvae were used for hemolymph extraction after surface sterilization with 70% alcohol for 1 min and 0.5% NaClO for 3 min inside a sterile hood. Hemolymph was collected in a sterile culture flask by clipping one of the fleshy prolegs. Hemolymph was treated to inhibit melanization using the method described by Grundschober et al. (1998). Cassava leaves were blended with distilled water and the mixture was heated at 65 °C for 35 min, 37 °C for 60 min, or not heat treated. The leaf extract samples were centrifuged at 2000 rpm for 10 min and the supernatant was then filter sterilized (0.2-μm Nalgene filter units) before being added to culture media.

Nolan's medium, a patented medium for *Entomophaga aulicae* (US patent number 5,728,572, 17 March 1998), was tested with the addition of 38.18 g/L sucrose and 0.7 g/L glucose. Media pH was corrected to 6.3–6.5 using 1 M NaOH or 1 N HCl.

2.3. Growth of *N. floridana* and *N. tanajoae* isolates in various cell culture media amended with serum

The ability of one *N. tanajoae* and two *N. floridana* isolates to grow in different commercially available liquid cell culture media was investigated. These isolates have different host ranges and distinct geographical origins and they were the only mite pathogenic isolates of *Neozygites* available in vitro during the time this study was conducted. *N. tanajoae* from Cruz das Almas, Brazil (BIN 8l13), was isolated from CGM; and *Neozygites* sp. (ARSEF 662) from the US and *Neozygites* cf. *floridana* (ARSEF 5376) from Colombia (both referred to here as *N. floridana*) were isolated from *T. urticae*. Hyphal bodies were produced in Grace's medium supplemented with 5% fetal bovine serum, 0.15% lactalbumin hydrolysate and 0.15% yeastolate (Gibco-BRL) and then washed in 200 mM sucrose. The inoculum was then seeded into 25 cm^2 culture flasks containing 3 mL of culture medium for a final concentration of 5×10^4 hyphal bodies/mL. The growth of the three isolates was compared in 5 base culture media (all manufactured by Gibco-BRL): Grace's insect medium, Express Five SFM, McCoy's 5A medium (modified), IPL-41 insect medium, and Sf-900 II SFM These media were variously supplemented with 5% fetal bovine serum with or without the addition of 0.3% lactalbumin hydrolysate + 0.3% yeastolate. Grace's medium supplemented with 5% fetal bovine serum and 0.15% lactalbumin hydrolysate + 0.15% yeastolate was also tested, totaling 11 types of culture media. Flasks were incubated on an orbital shaker at 75 rpm, 23 ± 1 °C, in the dark. The number of hyphal bodies was determined using a hemacytometer at 2 days intervals over 12 days. Four replicates were used with three replicates being conducted at the same time and another replicate conducted later. The effect of culture media and isolates as dependent variables on the maximum density of hyphal bodies were tested using general linear model. The maximum hyphal body densities were analyzed using one-way ANOVA and Tukey's HSD pairwise comparisons test to compare growth among isolates in each medium.

Using Brazilian isolates from Cruz das Almas (BIN 8l13, 8ddc) and Garanhuns (BIN 30) studies were conducted to test whether in vitro-grown isolates maintained their ability to produce conidia and were still pathogenic. Formation of primary conidia and capilliconidia from hyphal bodies of *N. tanajoae* was tested after cultures were maintained on a shaker at 90 rpm, 24 °C for six days in the dark. Cultures were then centrifuged and the hyphal bodies spread onto Petri dishes with 1% water-agar. The Petri dishes were maintained upside down at 24 °C in the dark until conidia were observed on the lid. CGM adult females were placed on Petri dish lids containing capilliconidia, and contamination was monitored under dissecting microscope. Mites with capilliconidia attached to the body were transferred to cassava leaf discs and maintained at the conditions used for in vivo production of *N. tanajoae* (Delalibera Jr., 2002).

2.4. Growth rate of *N. floridana* and *N. tanajoae* isolates in serum free media

Hyphal bodies of *N. tanajoae* from Cruz das Almas, Brazil (BIN 8l13), and *N. floridana* isolates from the US

and Colombia were produced in IPL-41 medium supplemented with 5% fetal bovine serum + 0.3% lactalbumin hydrolysate + 0.3% yeastolate (Gibco-BRL) and then washed in 200 mM sucrose. In the first bioassay, Grace's insect medium and IPL-41, both supplemented with 0.3% lactalbumin hydrolysate + 0.3% yeastolate, were tested using tryptic soy broth as a replacement for fetal bovine serum at 0.8, 1.67, and 2.0% (v/v). Grace's medium with calcium caseinate at concentrations of 0.13% and 0.4% was also evaluated with tryptic soy broth adjusted to 1.6% or 0.8%, respectively. Medium pH was corrected to 6.3–6.5 using 1M NaOH or 1N HCl. Grace's + 0.3% lactalbumin hydrolysate + 0.3% yeastolate was used as a control. Cultures were transferred at least twice in the medium being tested before cell growth was quantified. Fungal isolates were subcultured every 5–7 days depending on the growth rate, placing about 0.3 mL of fungal culture in 2.7 mL of new medium. Hyphal body densities were determined using a hemacytometer at 2 days intervals over 12 days.

In a second assay, combinations of the best cell culture media from previous experiments, Sf-900 II SFM and IPL-41, were tested without the addition of fetal bovine serum. Sf-900 II SFM was tested with and without supplementation of 0.3% lactalbumin hydrolysate + 0.3% yeastolate and also with 10% McCoy's 5A + 10% IPL-41. IPL-41 with 0.3% lactalbumin hydrolysate + 0.3% yeastolate was tested alone or in addition to 20% McCoy's 5A or with 50% Sf-900 II SFM. The maximum hyphal body densities and time to reach maximal density for all media combinations and isolates were analyzed using a general linear model.

2.5. Comparison of hyphal body morphologies

Hyphal bodies of 9 *N. tanajoae* and 2 *N. floridana* isolates were inoculated into IPL-41 media supplemented with 0.3% lactalbumin hydrolysate + 0.3% yeastolate to a final concentration of 5×10^4 hyphal bodies/mL. Length, width and number of nuclei were measured after 3 days on all isolates and also after 1 and 6 days for *N. floridana* isolates 662 and 5376 and for Brazilian *N. tanajoae* isolates from Cruz das Almas, BA (BIN 8l13) and Garanhuns, PE. Samples were stained using 0.8 μL of propidium iodide (Sigma Chemical) solution (3 mg/mL) per 1 mL of fungal culture and small drops of stained cultures were mounted on microscope slides and maintained at −80 °C until evaluation. Measurements were taken on 50 hyphal bodies per isolate using a fluorescence microscope with an excitation filter of 450–490 nm (Chroma blue filter) and a barrier filter of 520 nm. Effect of day of measurement and isolate on the length, width, volume and number of nuclei of hyphal bodies were analyzed by a general linear model and post hoc comparisons using Tukey's HSD pairwise tests. Cell volume was calculated by approximation to the nearest simple geometrical shape: cylinder. The association of number of nuclei and cell volume was assessed by linear regression using all measurements for all fungal isolates.

3. Results

3.1. Isolation and establishment of *N. tanajoae* in vitro

No growth of *N. tanajoae* was observed either in Nolan's medium or in any combination of Grace's with sucrose, heart infusion, tryptic soy broth, treated gypsy moth hemolymph, or cassava leaf extract. The first in vitro growth of any *N. tanajoae* isolate was obtained using Grace's medium supplemented with 5% fetal bovine serum + 0.3% lactalbumin hydrolysate and 0.3% yeastolate. However, most samples failed to grow in this medium, and the ones that were able to grow developed smaller, more rounded cells than hyphal bodies in mites, and these cultured cells degenerated after repeated transfers.

Healthy and long-term growth of *N. tanajoae* was first achieved using IPL-41 + 5% fetal bovine serum + 0.3% lactalbumin hydrolysate and 0.3% yeastolate. Using this medium many cultures of *N. tanajoae* were established and could be used to compare different media. Although isolates from Cruz das Almas, Garanhuns and Gravatá were maintained in culture for more than 1 year using IPL-41 + 5% fetal bovine serum + 0.3% lactalbumin hydrolysate + 0.3% yeastolate, the *N. tanajoae* isolates from Cotonou (BIN 35), Cristinápolis (BIN 12), Tianguá (BIN 10), Teotônio Vilela (BIN 14) and São M. dos Campos (BIN 15) required this same medium, except with 10% fetal bovine serum to sustain long-term growth. All isolates can be repeatedly subcultured using this medium with 10% fetal bovine serum.

Formation of primary conidia and capilliconidia from hyphal bodies was induced after 2–7 days when cells were spread onto water-agar. Capilliconidia produced in vitro in this way infected and mummified CGM, although virulence was very low (<10%).

3.2. Growth of *N. floridana* and *N. tanajoae* isolates in various cell culture media amended with serum

When *N. tanajoae* isolates from Brazil were inoculated in 11 different cell culture media, hyphal body densities increased in all media except Express Five and McCoy 5A media. Hyphal body densities greater than 10^7 hyphal bodies/mL were only observed in IPL-41 + 5% fetal bovine serum + 0.3% lactalbumin hydrolysate + 0.3% yeastolate and Sf-900 II SFM + 5% fetal bovine serum (Table 2). Production of *N. tanajoae* hyphal bodies was fastest and most consistent in IPL-41 medium + 5% fetal bovine serum + 0.3% lactalbumin

Table 2
Comparative growth of two *N. floridana* isolates from North Carolina (ARSEF 662) and Palmira (ARSEF 5376) and a *N. tanajoae* isolate from Cruz das Almas (BIN 8l13) in 9 cell culture media amended with 5% fetal bovine serum

Medium (+5% FBS)	Maximum density of hyphal bodies/mL ± SE ($\times 10^5$)		
	N. tanajoae	*N. floridana*	
	Cruz das Almas	Palmira	*N. Carolina*
Grace's	5 ± 4b	16 ± 3ab	29 ± 4a
Grace's + 0.15% LY	13 ± 8b	56 ± 3a	51 ± 2a
Grace's + 0.3% LY	8 ± 5b	51 ± 5a	65 ± 6a
IPL-41	19 ± 7b	54 ± 20ab	110 ± 12a
IPL-41 + 0.3% LY	159 ± 5a	177 ± 8a	155 ± 21a
Sf-900	103 ± 50b	236 ± 22a	229 ± 10a
Sf-900 + 0.3% LY	2 ± 1b	313 ± 42a	224 ± 27a
McCoy's	0 ± 0a	3 ± 3a	35 ± 26a
McCoy's + 0.3% LY	88 ± 33a	70 ± 21a	68 ± 15a

Means followed by the same letter in the same row are not significantly different from each other at $p < 0.05$. FBS, fetal bovine serum; 0.3% LY = 0.3% lactalbumin hydrolysate + 0.3% yeastolate. Cultures were inoculated with 5×10^4 hyphal bodies/mL.

hydrolysate + 0.3% yeastolate (Fig. 1). This medium yielded 1.53 (± 0.08) $\times 10^7$ hyphal bodies/mL after 8 days. At day 8, hyphal body densities were significantly different across media ($F_{8,25} = 35.1$, $p < 0.001$) and cell concentration in IPL-41 + 5% fetal bovine serum + 0.3% lactalbumin hydrolysate and 0.3% yeastolate was higher than any other medium. Maximum hyphal body densities on McCoy's 5A medium supplemented with 5% fetal bovine serum + 0.3% lactalbumin hydrolysate and 0.3% yeastolate and Sf-900 II SFM + 5% fetal bovine serum were observed after 10 and 12 days, respectively. With repeated subculture using these media, *N. tanajoae* cultures declined.

Growth of *N. floridana* and *N. tanajoae* isolates were observed in most cell culture media (Table 2). However,

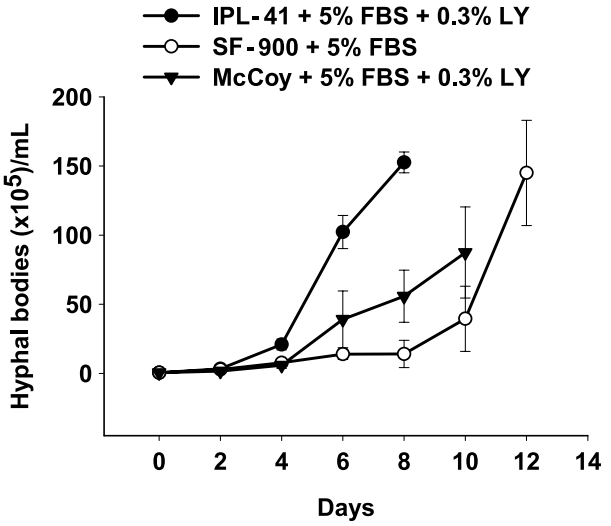

Fig. 1. Growth of hyphal bodies (mean ± SE) of *Neozygites tanajoae* from Cruz das Almas, BA, Brazil (Bin 8113) in three cell culture media. Only the lag and exponential phase part of the growth curves are presented. FBS, fetal bovine serum; 0.3% LY = 0.3% lactalbulin hydroltsate + 0.3% yeastolate.

different media were optimal for individual isolates ($F_{16,77} = 9.43$, $p < 0.001$). None of the isolates grew in Express Five medium + 5% fetal bovine serum alone or with supplements, and this medium was not included in the statistical analysis. The *N. tanajoae* isolate produced fewer hyphal bodies on four media than the *N. floridana* isolate from Palmira, 5376, and lower densities in six media compared to the North Carolina isolate, 662 ($F_{2,9} = 6.7$–30.3, $p < 0.025$). *N. tanajoae* did not produce higher densities of cells than *N. floridana* isolates in any tested medium. The two *N. floridana* isolates produced more than 2×10^6 hyphal bodies/mL in 7 and 9 out of the 11 media tested, respectively. *N. tanajoae* isolates reached hyphal body densities greater than 2×10^6/mL in only three media. Cell densities of *N. tanajoae* also increased slower than for *N. floridana* isolates in most media. On average, the *N. tanajoae* isolate reached 10^6 hyphal bodies/mL after 8.8 ± 0.6 days, and the isolates of *N. floridana* yielded the same numbers of cells after 7.6 ± 0.4 and 7.1 ± 0.2 days, respectively. The best medium for the Palmira isolate was Sf-900 II SFM + 5% fetal bovine serum + 0.3% lactalbumin hydrolysate + 0.3% yeastolate ($F_{8,26} = 30.3$, $p < 0.001$). This medium and Sf-900 II SFM + 5% fetal bovine serum provided the highest yield of hyphal body for the North Carolina ($F_{8,26} = 25.5$, $p < 0.001$). Although the medium Sf-900 II SFM + 5% fetal bovine serum + 0.3% lactalbumin hydrolysate + 0.3% yeastolate yielded high hyphal body densities of *N. floridana* isolates it was not optimal for *N. tanajoae*. For both species, most isolates reached optimal growth in 6–10 days and cell numbers declined after 10–12 days.

The two media yielding optimal growth of all three isolates were IPL-41 + 5% fetal bovine serum + 0.3% lactalbumin hydrolysate + 0.3% yeastolate and Sf-900 II SFM + 5% fetal bovine serum, but for the second of these media *N. tanajoae* produced statistically fewer cells ($F_{2,8} = 6.7$, $p = 0.02$). The *N. floridana* isolates did not

differ in production of hyphal bodies in all media tested. Although the final concentration of hyphal bodies produced by *N. floridana* isolates were not statistically different using Sf-900 II SFM + 5% fetal bovine serum alone or with 0.3% lactalbumin hydrolysate and 0.3% yeastolate, cell multiplication was faster in Sf-900 II SFM + 5% fetal bovine serum, reaching maximum cell density at day 6 (data not presented).

3.3. Growth rate of *N. floridana* and *N. tanajoae* isolates in serum free media

All serum free media tested failed to support continuous production of *N. tanajoae* isolates. The final concentration of *N. floridana* hyphal bodies was not affected by the deletion of fetal bovine serum in Grace's + 0.3% lactalbumin hydrolysate and 0.3% yeastolate ($F_{3,10} = 5.4$, $p = 0.176$) but the growth rate was two times slower ($F_{3,10} = 126.5$, $p < 0.001$). The isolate from North Carolina reached 4 (± 0.7) $\times 10^6$ hyphal bodies/mL after 12 days in the serum free medium and 5.8 (± 0.7) $\times 10^6$ hyphal bodies after 6 days when serum was added. Tryptic soy broth, pure or amended with calcium caseinate, negatively affected the growth of *N. floridana*. Maximum yields of hyphal bodies in Nolan's medium were 7.8 (± 1.7) $\times 10^6$ /mL after 11.6 ± 0.4 days for the *N. floridana* isolate from North Carolina and 7.9 (± 0.4) $\times 10^6$/mL after 11 ± 0.4 days for the isolate from Palmira.

All serum free cell culture media tested using Sf-900 II SFM or IPL-41 as the base medium supported production of $>10^7$ hyphal bodies/mL (Table 3) after 6–8 days, and cell densities were not statistically different among isolates ($F_{1,24} = 0.13$, $p = 0.725$) and media ($F_{5,24} = 1.74$, $p = 0.165$). Sf-900 II SFM medium without any supplementation supported growth of the *N. floridana* isolates of up to $>1.8 \times 10^7$ hyphal bodies/mL after 7.3 ± 0.7 days. Although not statistically significant, there was a tendency for increased hyphal body densities when supplementing IPL-41 with other cell culture medium components but this trend was not seen for Sf-900 II SFM.

3.4. Comparative hyphal body morphologies

In general, *N. tanajoae* hyphal bodies were shorter and thicker than those of *N. floridana* isolates. After 3 days of growth using measurements from 9 *N. tanajoae*

Table 3
Growth of *N. floridana* isolates from North Carolina (ARSEF 662) and Palmira (ARSEF 5376) in serum free cell culture media: maximum density of hyphal bodies and time to reach maximum density (mean ± SE)

Cell culture media	Hyphal bodies ($\times 10^5$)/mL		Time (days)	
	Palmira	N. Carolina	Palmira	N. Carolina
Sf-900	222 ± 67	185 ± 28	7.3 ± 0.7	7.3 ± 0.7
Sf-900 + 10% McCoy's + 10% IPL-41	162 ± 27	178 ± 14	6 ± 0	6 ± 0
Sf-900 + 20% McCoy's	162 ± 30	202 ± 33	6 ± 0	6 ± 0
IPL-41 + 0.3% LY	116 ± 40	139 ± 18	7.3 ± 0.7	7.3 ± 0.7
IPL-41 + 20% McCoy's + 0.3% LY	182 ± 16	153 ± 8	7.3 ± 0.7	7.3 ± 0.7
IPL-41 + 50% Sf-900 + 0.3% LY	189 ± 11	213 ± 17	8 ± 0	7.3 ± 0.7

All comparisons of hyphal body production and days among culture media within and between isolates were not statistically different ($p < 0.05$). 0.3% LY = 0.3% lactalbumin hydrolysate + 0.3% yeastolate.

Table 4
Size (mean ± SE; $n = 50$) and number of nuclei of hyphal bodies produced in vitro of two *N. floridana* isolates and eight *N. tanajoae* isolates for measurements taken 3 days after transfer to new medium

Isolate	Length (μm)	Width (μm)	Cell volume (mm^3)	Number of nuclei
N. floridana				
North Carolina (ARSEF 662)	43.6 ± 1.6a	7.3 ± 0.2a	1.90 ± 0.12bc	3.8 ± 0.1cde
Palmira (ARSEF 5376)	42 ± 1.6ab	7.3 ± 0.2a	1.75 ± 0.11c	3.6 ± 0.1cde
N. tanajoae				
Cruz das Almas (BIN 8l13)	37.9 ± 1.5bc	8.2 ± 0.2ab	2.04 ± 0.11bc	3.3 ± 0.1e
Garanhuns (BIN 30)	37.9 ± 1.7bc	8.7 ± 0.2bc	2.27 ± 0.13bc	3.2 ± 0.1e
Cotonou (BIN 35)	32.9 ± 1.3d	8.7 ± 0.2bc	2.01 ± 0.14bc	3.7 ± 0.2cde
Cruz das Almas (BIN 8ddc)	29 ± 1.3def	10.4 ± 0.2ef	2.49 ± 0.16b	6.2 ± 0.3b
Cristinápolis (BIN 12)	26.2 ± 1.0fgh	9.7 ± 0.2cde	1.94 ± 0.10bc	4.2 ± 0.1cd
Gravata (BIN 16)	31.1 ± 1.1de	9 ± 0.2bcd	2.00 ± 0.12bc	4.4 ± 0.2c
Tianguá (BIN 10)	25.7 ± 1.1gh	9.7 ± 0.5cde	1.94 ± 0.17bc	3.5 ± 0.1de
Teotonio Vilela (BIN 14)	23.8 ± 0.9h	10.1 ± 0.2de	1.94 ± 0.11bc	3.8 ± 0.2cde
Sao M. dos Campos (BIN 15)	33.6 ± 1.2cd	11.6 ± 0.3f	3.57 ± 0.19a	7.3 ± 0.3a

Means followed by the same letter in the same column are not significantly different from each other at $p < 0.05$.

isolates, only the isolates of *N. tanajoae* from Cruz das Almas had both length and width ranges similar to *N. floridana* isolates and the length of hyphal bodies of the isolate from Garanhuns was similar to Palmira (Table 4). More detailed comparisons among 4 isolates during different growth phases revealed that hyphal bodies of the two *N. floridana* isolates are longer than *N. tanajoae* isolates from Cruz das Almas and Garanhuns one day after inoculation ($F_{3,196} = 29.5$, $p < 0.001$). Although not statistically significant, *N. tanajoae* hyphal bodies also tend to be shorter than *N. floridana* after 3 and 6 days post inoculation (Fig. 2). The average hyphal body length of *N. floridana* isolates from N. Carolina and Palmira for all three dates was 46.3 ± 1 and 45.9 ± 1.2 μm and hyphal body length for *N. tanajoae* isolates from Garanhuns and Cruz da Almas were 39.2 ± 0.9 and 36.1 ± 0.8 μm, respectively.

Hyphal body lengths were found to change with time ($F_{2,588} = 50.1$, $p < 0.001$), becoming shorter. The length of hyphal bodies of isolates from North Carolina and Cruz das Almas ranged from 52.1 ± 1.9 and 37.6 ± 1.3 μm after 1 day, to 43.2 ± 1.6 and 32.9 ± 1.1 μm after 6 days, respectively. Cell width was not statistically different at different times ($F_{2,588} = 2.9$, $p = 0.057$) and no consistent differences were observed among the two *N. tanajoae* and the two *N. floridana* isolates.

The number of nuclei varied between isolates ($F_{3,588} = 16.3$, $p < 0.001$) and also changed during evaluations ($F_{2,588} = 66.7$, $p < 0.001$). Interaction between isolates and days was not significant ($F_{6,588} = 1.5$, $p < 0.177$). The numbers of nuclei were greater one day after being transferred to the new IPL-41 supplemented medium than after 3 days and, also was greater than after 6 days.

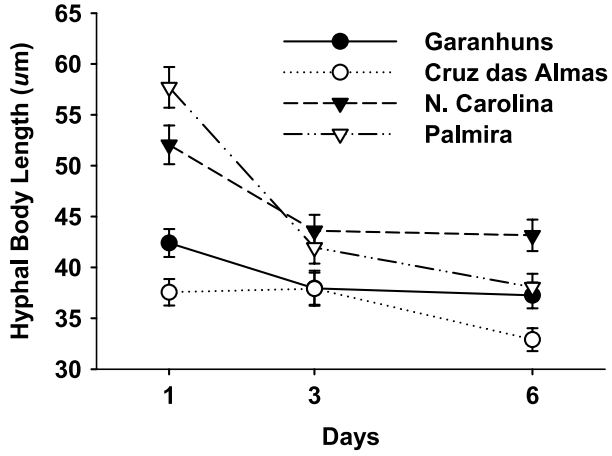

Fig. 2. Variation of hyphal body length (mean ± SE) during growth of cultures of *Neozygites floridana* isolates from North Carolina (ARSEF 662) and Palmira (ARSEF 5376) and *N. tanajoae* isolates from Cruz das Almas (BIN 8l13) and Garanhuns (BIN 30) in IPL-41 media + 5% FBS + 0.3% lactalbumin hydrolysate + 0.3% yeastolate.

The average numbers of nuclei of the isolates from North Carolina and Cruz das Almas ranged from 5 ± 0.3 and 3.7 ± 0.2 after 1 day, when a high proportion of cells were in mitosis, to 3.4 ± 0.1 and 2.9 ± 0 after 6 days, respectively. At day 6, when cell multiplication was lower, the *N. floridana* isolate from North Carolina had 6% hyphal bodies with 2 nuclei, 58% with 3 nuclei and 32% with 4 nuclei. The *N. floridana* isolate from Palmira was very similar with 6, 54, and 32% of the hyphal bodies with 2, 3 and 4 nuclei, respectively. The proportion of cells containing 2, 3 and 4 nuclei of *N. tanajoae* isolates from Cruz das Almas and Garanhuns were 12, 88, and 0%, and 16, 78, and 6%, respectively, after 6 days.

The *N. tanajoae* isolates from Cruz das Almas (BIN 8l13) and from Garanhuns did not differ from each other in the number of nuclei or cell size at any date. However, variations in the morphology of hyphal bodies in vitro were observed among other *N. tanajoae* isolates. The number of nuclei per cell of all *N. tanajoae* isolates ranged from 2 to 14, but most cells contained 3–5 nuclei. Isolates from Tianguá, Cristinápolis, Teotônio Vilela, Cotonou and Gravatá were predominantly 3–4 nucleate. The proportions of hyphal bodies with 3 and 4 nuclei for these isolates was greater than 60%. If we consider the cells with 6 and 8 nuclei per cell as being in the process of dividing (post mitotic but before cell division), this proportion would range from 73% for the isolate from Cristinápolis to 100% for the Cotonou isolate. Isolates São Miguel dos Campos and Cruz das Almas (8ddc) contained more nuclei per cell than others. Forty percent of hyphal bodies of the isolate from Cruz das Almas contained 5 or 10 nuclei. The percentage of cells with 5 or 10 nuclei and cells with 7 or 14 nuclei was 22% and 32% for the isolate from São Miguel dos Campos, respectively.

The cell volume of *N. floridana* isolates is similar to most *N. tanajoae* isolates (Table 4). Hyphal body volume of the *N. tanajoae* isolate from São Miguel dos Campos is greater than all other isolates. Isolates with more nuclei also presented greater cell volume ($F_{1,549} = 211.1$; $p < 0.001$). However, only 27.7% (R^2 adj) of the variation on cell volume can be attributed to the number of nuclei.

4. Discussion

In this study a culture medium was developed for production of hyphal bodies of the fungus *N. tanajoae*. For the sake of convenience we will refer henceforward to this preferred medium for *N. tanajoae*, IPL-41 + 5% fetal bovine serum + 0.3% lactalbumin hydrolysate + 0.3% yeastolate, as NT-1. Using methods, media and supplements commonly used for cell culture, we were able to establish several isolates of *N. tanajoae* in

vitro. While some cultures were started in only 3 weeks other cultures were dormant for months before growth occurred. Lynn (1989) affirmed that cell culturists commonly observe this delay and cautioned that patience may be the most powerful tool in developing insect cell lines. We demonstrated that different insect cell culture media yield high production of hyphal bodies of isolates by *N. floridana*, and we think it is reasonable to suggest that this technology may be useful in the isolation and production of other fastidious entomopathogenic fungi.

Insect hemolymph formed the basis for the earliest insect cell culture maintenance media (Goldschmidtt, 1915), and this is still a major ingredient for production of *N. parvispora* (Grundschober et al., 1998) but insect hemolymph (from gypsy moth) did not help to establish continuous cultures of *N. tanajoae*. The first cell lines from insects were developed using a medium modified by Grace (1962), initially supplemented with insect hemolymph and later with fetal bovine serum. Grace's medium with fetal bovine serum was successfully used for growth of protoplasts of *Entomophthora aulicae* (= *E.egressa*) (Tyrrell and MacLeod, 1972) and since then became an important basal medium for in vitro production of some other entomophthoralean fungi (Dunphy and Nolan, 1979; Freimoser et al., 2000; Grundschober et al., 1998; Latgé and Beauvais, 1987; Leite et al., 2000; Soper et al., 1988). Since Grace's medium was so widely used for *Neozygites* spp. and other entomophthoralean species, the first approach we took to develop a culture medium for *N. tanajoae* was to supplement this basal medium with ingredients that have been proved to increase growth of other entomopathogenic fungi (e.g., heart infusion, tryptic soy broth, lactalbumin hydrolysate and yeastolate). We also tested if insect hemolymph as well as cassava leaf extract could provide growth factors not present in the defined ingredients used. Despite its utility for other fungi, supplemented Grace's medium did not support continuous growth of *N. tanajoae* isolates.

The IPL-41 insect medium was developed by Weiss et al. (1981) as a modification of Goodwin's original IPL formulation (Goodwin and Adams, 1978). It was designed for the growth of cells from lepidopteran species, specifically *Spodoptera frugiperda*. This medium has also been used for in vitro rearing of the aphid parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae) (Digilio, 1999). We believe this is the first time that either IPL-41 or Sf-900 II SFM has been tested for in vitro growth of entomopathogenic fungi.

Until the late 1980s, the cell culture media widely used, whether for mammalian, avian, fish or insect cell lines required supplementation with fetal bovine serum (Digilio, 1999). More recently, some serum replacements have been described for specific cell lines (Goodwin, 1991; Inlow et al., 1989). In the process of developing an inexpensive medium for mass production of *Entomophaga aulicae* hyphal bodies, Dunphy and Nolan (1982) started from a simplified medium that required fetal bovine serum until Nolan (1993) was able to elaborate a defined medium that replaced fetal bovine serum with 0.8% tryptic soy broth and 0.4% calcium caseinate. When we supplemented Grace's and IPL-41 media with tryptic soy broth and calcium caseinate and tested Nolan's medium for production of all *N. tanajoae* isolates we observed no growth in any of these media. Growth of *N. floridana* was negatively affected by addition of tryptic soy broth and calcium caseinate, but this fungus was able to grow in Nolan's basal medium. IPL-41 and Sf-900 are cost-effective, serum-free formulations developed for some cell lines and proved to be good as basal media for *N. floridana* and *N. tanajoae*. Although *N. tanajoae* still requires fetal bovine serum for continuous growth in these media, both provided very good yields of hyphal bodies of the two *N. floridana* isolates without adding fetal bovine serum. Combinations of IPL-41 and Sf-900 II SFM serum-free media supported production of hyphal bodies of *N. floridana* from 2.1 to 4.4 times greater than in Grace's medium + 5% fetal bovine serum + 0.3% lactalbumin hydrolysate + 0.3% yeastolate used in previous studies with *Neozygites*.

We demonstrated that a remarkable difference in nutritional requirements exists between *N. tanajoae* and *N. floridana*. *N. tanajoae* is a particularly fastidious fungus while *N. floridana* grows in a broader range of media. Actually, Express Five medium was the only medium that did not support any growth of *N. floridana* isolates. This medium does not contain L-glutamine, an essential amino acid, and this omission may account for this result. *N. tanajoae* also differs morphologically from the two *N. floridana* isolates. *N. tanajoae* isolates produced smaller hyphal bodies in supplemented IPL-41 medium.

The size of *N. tanajoae* hyphal bodies was relatively similar to data presented in two other studies on in vitro cultures of *Neozygites* spp. Grundschober et al. (1998) reported that hyphal bodies of *N. parvispora* were an average length of 27 ± 1 μm (varying from 11 to 46 μm) and width 6 ± 0.1 μm (varying from 3.9 to 7.8 μm). Butt and Humber (1989) observed that the mean cell length of *N. floridana* increased from 29.8 ± 8.3 μm (interphase) to 38.8 ± 4.2 μm (metaphase) while the diameter remained unchanged. We reported that the average cell length decreased as cultures aged and mitosis became less common. Since each isolate reaches exponential growth at different times in different media, only measurements taken at different phases of culture growth give a more accurate profile of cell sizes for isolate comparisons. On the other hand, counts of nuclei in hyphal bodies are more stable and representative of interphase cells after 6 days, when mitosis is drastically reduced and cells started to degenerate.

Neozygites tanajoae is a fungus whose cells typically contain 3–5 nuclei, except for one isolate from São M. dos Campos. For this isolate a greater proportion of the cells contained 7, 8, and 10 nuclei. These unusual numbers of nuclei may be characteristic of this strain. We observed that in vivo, 95% of hyphal bodies ($n = 652$) of 10 infected mites from São M. dos Campos contained 5 nuclei and 4% contained 10 nuclei (data not shown). Abnormal numbers of nuclei could be generated by the physiological stress caused by adaptation to in vitro cultivation. This hypothesis was also suggested by Grundschober et al. (1998) to explain the unusual nuclear number of *N. parvispora* nuclei in vitro (2–8 nuclei). The number of nuclei of *N. tanajoae* is in the same range as other mite pathogenic species of *Neozygites*: for example *N. acaricida* = 3–4 nuclei per cell and *N. floridana* = 3–5 nuclei (Keller, 1997).

The distinction of *N. tanajoae* from *N. floridana* was initially proposed based on host specificity, genetic and physiological patterns and is supported by the results presented in this study. *N. tanajoae* is a particularly fastidious species highly specific to CGM and grows well in few media while *N. floridana* which is less host specific, grows in a broader range of media, including serum free media.

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